Methods in Pharmacology and Toxicology

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In Vitro Toxicology Systems

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Humana Press

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ISSN 1557-2153 ISSN 1940-6053 (electronic)
ISBN 978-1-4939-0520-1 ISBN 978-1-4939-0521-8 (eBook)
DOI 10.1007/978-1-4939-0521-8
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2014935368

Printed on acid-free paper

Humana Press is a brand of Springer
Springer is part of Springer Science+Business Media (www.springer.com)

Foreword

Laboratory animals have long been used as surrogates for human beings, because it has been considered acceptable to expose them to conditions and procedures which would not be considered acceptable, if applied to ourselves.

Initially, the focus was mainly on gaining a better understanding of how the body's cells, organs, and systems function and are controlled, and how failures of one kind or another can lead to pathological conditions. Then, from about the middle of the last century, animals came to be used more and more in tests to determine the effects of exposure to chemicals and chemical products—in attempts to determine information of direct relevance concerning the efficacy of drugs and vaccines and/or the adverse effects of chemicals and other kinds of chemical products, in the hope of predicting likely effects in humans, as a basis for appropriate risk assessment and risk management.

However, attitudes toward the reliance on this approach are now changing, with the increasing recognition that the knowledge gained from animal studies cannot be expected to have direct relevance to humans, but can even have dangerous consequences.

This problem is particularly acute for the pharmaceutical industry, which is in a state of crisis because of the increasing occurrence of the late withdrawal of new drugs as a result of lack of efficacy or unacceptable side effects not detected during preclinical testing, despite the application of highly expensive and seemingly sophisticated testing in animals. Meanwhile, the introduction of the EU REACH system for chemical toxicity has revealed that many more chemicals than had been expected lack the information needed to provide for what is considered to be an acceptable risk assessment. Also, it has to be recognized, albeit very reluctantly in some quarters, that two of the main types of animal test, which are very costly and which can cause great suffering to the animals involved, namely, reproductive toxicity tests and the rodent bioassay for chemical carcinogens, simply cannot be relied on to identify chemicals likely to have adverse effects during human reproduction or to cause cancer in humans. I have never understood how the full lifetime feeding of the maximum tolerated dose of a chemical to a rat or mouse could tell us anything about the carcinogenicity of the chemical for rodents, let alone for humans. We don't eat very high doses of single chemicals throughout our lives.

There are two main and insuperable reasons for these difficulties. First, functions and controls in animals and humans tend to be very different in detail, however similar they may appear to be on the surface. Animals are highly adapted to their individual and specific lifestyles and environments. During evolution, species separate and diverge from common ancestors, based on these adaptations, which tend not to involve the emergence of something totally new, but which rely instead on modifications of what was already there. This has profound implications for attempts to model human diseases in animals, especially since, in view of the absence of sufficient knowledge about what is being modeled, it is impossible to judge whether or not a particular model has any value.

Russell and Burch referred to the problem of species difference in *The Principles of Humane Experimental Technique* (1959), when they warned of the "high fidelity fallacy."

This is the assumption that, because other mammals are similar to humans in many respects, they are always the best models to use in fundamental biomedical research, drug development, and toxicity testing, where humans are the focus of concern. This warning has been largely ignored.

One response to the unsolvable problem of species differences is the attempt to humanize animals by transferring human genes into their genomes, in the hope of simulating effects and responses in humans. However, that can be considered naïve, since the manipulation of complex networks of interacting controls, which are not sufficiently well understood and which will inevitably differ considerably in animals and humans, is likely to produce information which cannot be interpreted with confidence, and which may be dangerously misleading.

The second insuperable problem is that the "human" being to be modeled in animals doesn't actually exist. Human polymorphism leads to an infinite variety of different humans—there are many, many subpopulations within the overall human population, which will differ in their susceptibilities to disease and in their responses to chemicals and chemical products, including drugs. One result of this is that a drug which is highly effective in one patient can be lethal to another. Similarly, a chemical which has no effect in some individuals can induce a highly allergic response in others.

The only way forward is to recognize that the modern, but still developing, techniques of cell biology and molecular biology, combined intelligently with the vast information storage and computational systems which are now available, should be applied directly to human material in vitro and ex vivo, and in some situations, subject to strict ethical controls, to human volunteers. Carefully planned and executed, this approach could take account of human polymorphism, past or concurrent disease, and the differential effects of age, occupation, lifestyle, and exposure to medicines and other chemicals.

In the pharmaceutical industry, for example, the "one drug suits all" concept has been overthrown, and therapies in the future will involve "personalized medication," where the treatment will be designed specifically for the individual patient. In the case of other chemicals and products, the "one test suits all" concept also needs to be abandoned, in terms of both effects and people, and replaced by "personalized safety evaluation," which takes account of hereditary and lifestyle factors.

The very challenging prospect, which is the subject of this most important book, is that human-based in vitro studies and procedures could make major contributions to the two seemingly insuperable problems, if handled critically and intelligently. The increasing array of in vitro systems includes the use of cell fractions, cell lines, stem cells (including induced pluripotent stem cells), engineered tissues, dynamic bioreactors, multiorgan systems, and cells-, organs-, and (even) humans-on-a-chip, combined with high-throughput screening, high-content screening, the "omics" approaches, systems modeling and simulation, pharmacokinetic and toxicokinetic modeling, virtual tissue modeling, virtual human populations, and biomarkers, strategically used, with effective bioinformatics support.

One problem, evident from the chapters in this book, is the vastness of the range of technical possibilities. Manageability will be a key factor in the way forward, combined with the need to rigorously and regularly evaluate the relevance and reliability of what is proposed or what is being done, including whether it will yield a clear and applicable outcome.

One trap which must be avoided is to use animal test data as the "gold standard" to be matched by nonanimal, replacement alternative tests. If the animal tests themselves are not sufficiently relevant and reliable, how can the data they provide be used in the validation of human-based (by definition, more relevant) tests? Sadly, this trap is often laid, not least by

some regulators, who say that they feel "more comfortable" with animal test data than with predictions based on in vitro procedures.

It is essential that the highest scientific standards should be made, through, for example, compliance with the principles of Good Cell Culture Practice, which is analogous to Good Laboratory Practice and Good Manufacturing Practice.

There is also a danger of overspecialization and isolation, leading to blind alleys and the pursuit of red herrings, and of the production of enormous amounts of data. Russell and Burch referred to this in *The Principles*, where they feared the "gradual growth of awe before experts," and said that "respect for expert specialist knowledge should never become uncritical." They added that "the problem of interspecialist communication merges into the general one of information retrieval" and that "we now have far too much information as a species to digest as individuals." What they foresaw is an even greater problem today, when we are overwhelmed with information which is not critically evaluated before it is dumped on the world. We even have electronic journals, which publish manuscripts as they are received, without independent peer review. What we need are more avowed synthesizers, capable of broad and lateral thinking, and not committed to a particular dogma or strategy.

In particular, there is a need to recognize that it is not possible to have "mechanistic tests" without sufficient knowledge of the "mechanism" on which the test purports to be based. That is why fundamental and applied toxicology must progress hand-in-hand, so that there is greater confidence that the right questions are being asked, before attempts are made to answer them.

It is also essential to make full use of bioinformatics and what is known as systems toxicology and evidence-based toxicology, as a way of developing and applying intelligent and integrated strategies involving stepwise approaches. One major concern is that the nonanimal test possibilities will become so numerous and so costly that their use will not be practicable. Decision-tree schemes will therefore be essential. For example, if the likelihood of major hepatotoxicity is revealed, it will not be essential to test for toxicity to the kidney or the thyroid gland, or for carcinogenicity or reproductive toxicity.

It is now almost exactly 50 years since I went as a postdoctoral fellow learn about cell culture in Harry Rubin's group in the Virus Laboratory at the University of California at Berkeley. The use of cell cultures had created the breakthroughs in quantitative animal virology, which led, *inter alia*, to the production of polio vaccines (albeit at the cost of the lives of hundreds of thousands of rhesus monkeys, whose kidney cells were used to produce the viruses for the vaccines). We worked on Rous sarcoma virus (RSV) and chicken leucosis viruses in chick embryo fibroblast cell cultures. Rubin and Temin had developed an assay for RSV, based on the production of foci of virus-transformed fibroblasts, and Temin did the crucial experiments which showed that RSV, an RNA virus, made a DNA copy of itself, which was used to produce new virus particles. The enzyme involved was reverse transcriptase, and its discovery was one of the most important leaps forward in cell and molecular biology.

At that time, the fibroblasts and kidney cells were mere substrates in which the viruses could replicate, and there were few signs of the outstanding and astounding developments in cell culture technology which would give us the impressive range of techniques and procedures which are available today. It could be said that the fundamental science of toxicology in general, and the applied science of toxicity testing in particular, have been painfully slow in adopting these developments to their benefit, largely due, no doubt, to the entrenchment of the animal experimentation bias. However, I am confident that the

regrettable situation is about to change, thanks to the huge efforts being invested in industry and in academia throughout the world.

I am greatly encouraged by the excellent chapters in this book. However, as Russell and Burch warned, there is a danger that the reader will be overwhelmed by the detail and the sophistication. One way to overcome that problem is to begin by reading only the Introductions of the various chapters, to see how well the authors have set the background scene of what is to come, before looking at their Discussions and Conclusions sections, to see how they summarize the current state of the art and look to the future. Then, an overall impression having been gained, the detailed coverage and supportive evidence in the middle of each chapter can be carefully scrutinized and appreciated.

Ultimately, the question is whether the various in vitro approaches will contribute to an increase in understanding and a decrease in uncertainty. We cannot escape the fact that, although we may have many pieces of information to try to fit together, we certainly don't have all the pieces we need and we don't know how many pieces are missing. In addition, we must be suspicious that the pieces we do have are not merely the parts of one puzzle, but may be parts of an unknown number of different puzzles. Moreover, we cannot assume that all the pieces are of equal value, as we know that, far from being lifeless equivalents cut from the original picture with a jigsaw to form a conventional jigsaw puzzle, there are, within each piece of information having potential pharmacotoxicological significance, stories and histories, and pluses and minuses, and main streams and blind alleys, and dynamic interactions among them that are far more profound. Worst of all, we have no picture on a box to guide us—we have to create the eventual picture or pictures ourselves, by using strategies and applying rules which we have to devise and validate along the way.

Nottingham, UK ***Michael Balls***

Preface

In this book, we attempt to bring together the important issues and considerations we believe are needed in order to develop a workable, reliable, integrated testing strategy for the replacement of animals in toxicity testing regimes.

We begin the book with a review on "The past, present, and future of chemical risk assessment" by Alice Limonciel. She describes the history of the development of chemical testing and the evolution of chemical regulation. This process has had several key milestones. Among them were Elixir Sulfanilamide in 1937 and the thalidomide disaster in the late 1950s and early 1960s. The unfortunate use of the solvent diethylene glycol in Elixir Sulfanilamide caused the deaths of over a hundred people due to acute renal failure and introduced regulations where proof of safety of a compound was required to be shown before marketing. The thalidomide disaster introduced the necessity to test for reproductive toxicity. It is perhaps not surprising that these two examples were pharmaceuticals.

While testing is important for the chemical and cosmetic industry, the pharmaceutical industry is somewhat a special case as compounds are designed to be taken up, distributed, and have biological activity. Thus testing is a necessary and highly regulated part of drug development. However, just because we rigorously test compounds doesn't mean we necessarily predict toxicities or a lack of them in humans. Individually, nonhuman mammals poorly predict human toxicity, and thus several species are used to cover predictive ground, unfortunately at the expense of specificity. Therefore, there is an inevitable loss of compounds which are toxic in animals but safe in humans. Thomas Hartung (contributor of Chap. 11) has pointed out that aspirin, one of the most widely used pharmaceuticals today, would have most likely not been brought to market if it had to pass through current preclinical testing regimes. Thus one of the main scientific rationales for developing in vitro alternatives is to improve on current animal-based testing regimes in the preclinical phase.

The ability to maintain cells outside the living body is documented as far back as 1885, when the zoologist Wilhelm Roux maintained embryonic chicken cells in a warm saline solution for several days. However, the true foundation of modern cell culture was arguably not until the mid 1950s when Eagle began to investigate the nutritional requirements of cells in culture [1, 2]. Already in 1959, Russell and Burch had realized the importance of cell culture as a real alternative to animal use, stating that "Mammalian tissue cultures have become one of the most important replacement techniques, and indeed one of the most important developments in biology" [3]. Since 1959, there has been a dramatic increase in the development and use of in vitro cell cultures which was mostly driven by technological advances in molecular biology such as polymerase chain reaction (PCR), transfection, and gene silencing. Primary cells and cell lines have now become extremely widely used tools. The improvements in cell immortalizations, such as telomerase overexpression [4], the development of high-content omic approaches, and the discovery of methods to make somatic cells pluripotent are continuing to push back the borders of in vitro research. These approaches are well suited to pharmacological and toxicological approaches and have great potential to increase our understanding of the molecular perturbations of chemicals and may eventually overtake animal studies as predictive tools.

The safety assessment of chemicals is a composite of hazard identification and risk of exposure. Thus for the pharmaceutical industry in particular, where the chemical is intended to enter the body and usually the circulation, all cells are potentially exposed making hazard identification of primary importance. If in vitro toxicity testing regimes are to replace whole animal tests for pharmaceutical development, they will have to represent all the major organs and tissues. While this might sound an impossible task, it could be more manageable by considering a tiered approach using the most commonly affected cells or tissues first. The first line are the liver and kidney, since, due to their respective roles in xenobiotic metabolism and excretion, they are exposed to and interact with a wide variety of chemical entities. So theoretically if a lead chemical demonstrated either hepatotoxicity or nephrotoxicity at concentrations close to their therapeutic range, they should be stopped at this stage. However, if this is not the case, other cell systems would then need to be tested. In vitro models for liver and kidney toxicity are discussed in Chaps. 2 and 4, respectively. The heart is obviously a vital organ and any compound that adversely affects its function could have very serious implications for health. Cardiotoxicity is the primary reason for postmarketing drug withdrawals and thus is of major interest for drug development [5]. The progress in the development of in vitro cardiac models is discussed in Chap. 3, and detailed protocols are provided. Neurotoxicity and injury to the blood brain barrier are also a major toxicological concern, particularly with the potential of chemical-induced injury to contribute to neurodegenerative diseases such as Alzheimer's and Parkinson's. The issues concerned and in vitro models available are detailed in Chaps. 6 and 7. The lung due to its involvement in blood oxygenation, metabolism, and the elimination of volatile substances is also an important toxicological target and is of special interest for drug delivery. The lung represents a selective barrier between the external and internal environments and is thus challenged on a permanent basis with air-borne pollutants including nanoparticles. In vitro models of the lung are reviewed in Chap. 5, while the special consideration of nanoparticles is addressed in Chap. 21. Xenobiotics have the potential to interfere with immune responses either by increasing or decreasing specific immune activity, and thus can lead to immunosuppression, sensitization, autoimmune disease, and may even promote cancer. The challenges associated with the development of immunotoxicity assays in vitro are discussed in Chap. 11.

The organs and tissues mentioned so far are important as their disturbance can lead to severe ill health and mortality. However, nonvital organs where quality of life can be severely impaired should also be considered for in vitro screening regimes. For example, many compounds, including aminoglycoside antibiotics, can cause permanent deafness, a situation which can have serious implications to life quality. The mechanism of ototoxicity and the in vitro models available are discussed in Chap. 9. Of special consideration for the cosmetic industry are the external physiological barriers and body surfaces, where cosmetics are often applied, for example the skin and eyes. Indeed the progress for the development of alternative nonanimal strategies has been most successful, so far, for dermal and ocular toxicity (Chaps. 8 and 10).

Many compounds either through direct action on DNA or indirect action, for example through chronic tissue injury, immunomodulation, and endocrine disruption, can cause cellular and tissue perturbations leading to the development of cancer. Thus, the carcinogenic potential of compounds is of critical importance for human safety. While several in vitro systems for testing genotoxicity are available, the identification of nongenotoxic carcinogens is more difficult. These issues are elaborated in more detail in Chap. 14.

In addition to effects of compounds on the intended individual, we also need to consider their impact on fetal development and reproductive potential. We now know that the

placenta is not an all exclusive barrier to the maternal environment. Certain chemicals, for example thalidomide, can cross this barrier, where they may cause serious adverse developmental effects. Chemicals, such as endocrine disrupters, may in addition reduce fertility which is a serious societal concern. Thus development and reproductive toxicity are important endpoints and are discussed in Chaps. 12 and 13.

As already mentioned, the fairly recent discovery of the possibility to induce pluripotency in somatic human cells (inducible Pluripotent Stem Cells, iPSC) [6], has the potential to revolutionize how we study human diseases and is likely to provide a plethora of new biological tools for pharmacological and toxicological investigations. Apart from providing a new source for primary cell culture, iPSC-derived target cells could form the first in vitro basis for studying population-based dynamics, genetic susceptibility, and idiosyncrasies. The development and use of iPSC for the major target organs is addressed in Chap. 15, while the use of iPSC and progenitor cells for neurodevelopmental toxicity is specifically reviewed in Chap. 16.

One of the major driving forces for the use of in vitro systems is their applicability to high-content analysis. Indeed the coupling of well-characterized relevant cell culture systems with powerful high-content, information-rich techniques such as transcriptomics, proteomics, metabolomics, and high-content imaging is pushing back the boundaries and allowing a true mechanistic understanding of molecular events (Chaps. 17 and 18) [7]. The use of these new technologies has provided us with a vast amount of mechanistic information on how cells function at a molecular level and how they deal with chemical and physiological stressors [8]. These types of experimental approaches are driving a new age in toxicological science where the focus is the discovery and elucidation of molecular mechanisms underlying chemical-induced cellular perturbations (Chap. 19). Indeed the OECD is promoting the development of the so-called "Adverse Outcome Pathways" (AOP) concept where a molecular initiating event, in which a chemical interacts with a biological target(s), is followed by a sequential series of events that ultimately result in an adverse outcome in an individual organisms or a population [9]. The elucidation of such molecular pathways relevant for adverse effects of compounds can lead us to the discovery of mechanistically anchored biomarkers. These biomarkers can be used to develop better predictive systems or may even be employed in clinical settings (Chap. 20).

A very important but often neglected aspect of in vitro toxicology is pharmacokinetics or toxicokinetics. Kinetics deals with how a test compound is altered by the system it is applied to. For an in vitro system, the available concentration of the compound can be decreased by binding to cell culture-ware such as a plastic cell culture dish, by binding to proteins in the cell culture medium, by evaporation and due to cellular uptake or cellular metabolism. The latter two points are critical in both in vitro and in vivo systems and are discussed in detail in Chap. 22. Knowing the actual concentration that cells can interact with, either by measurement as a free concentration in the cell medium or as a tissue concentration in the cell lysate, is crucial not only for experimental interpretation but also to extrapolate to the in vivo situation. Indeed, we must eventually extrapolate from in vitro to in vivo in order to establish safe exposure limits, which is after all the end goal of the exercise. These issues are dealt with in Chaps. 23 and 24.

In order to realize the vision of Russell and Burch and to go a step closer to animal free testing, we will require an integrated, systems biology approach, utilizing good cell culture practice [10], good laboratory practice, relevant and robust biological systems together with appropriate analytical tools and prediction models. Such an integrated strategy should be fit-for-purpose and need to be recognized and accepted by regulatory authorities. Thus

it is of utmost importance that scientists, industry, and regulators understand the needs of each other; only then can an integrated, tiered strategy based on in vitro techniques be put in place. In Chap. 25, the considerations in the development of in vitro toxicity testing methods intended for regulatory use are detailed.

In conclusion, the majority of the contributors of this book share our opinion that the use of animals for safety assessments is approaching its end of life and will eventually be phased out by more predictive human-derived in vitro systems and *in silico* approaches. What these in vitro systems will be like is uncertain, but we would be surprised if iPSCs were not an integral part of it, as this technology allows both human population-based screening and safety evaluation tailored to individuals. Finally, we were delighted to receive such a positive response from the experts we contacted and were very pleased that, without exception, each chapter was written with the high standards and expert insight that we hoped for. We are confident that this book has accomplished its goals and will be of benefit not only to students, scientists, and regulators working in the field of chemical safety assessment but also to the wider scientific audience.

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